#13 Attach

PATENT
38-21(10525)A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

considered 4/14/91 DF

In Re The Application Of:	)	
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ROBERT T. FRALEY and STEPHEN G. ROGERS	)	Group Art Unit:
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Serial Number:	)	
	)	Examiner:
Filed: December 7, 1990	)	
	)	
Title: CHIMERIC GENES FOR TRANSFORMING PLANT CELLS USING VIRAL PROMOTERS	)	December 7, 1990

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Robert B. Horsch, declare that I am a United States citizen and a resident of St. Louis County, Missouri; that I am a research biologist by profession, having been graduated by the University of California, Riverside, with a degree of Doctor of Philosophy in Genetics in 1979. I further declare that since 1981 I have been employed by Monsanto Company in plant molecular biology research and more specifically in the area of Crop Transformation and that my present title is Manager, Crop Transformation Group. Prior to accepting employment with Monsanto, I was a Postdoctoral Fellow at the University of Saskatchewan involved in genetics research. I also declare that I am presently an Adjunct Professor in the Department of Biology at Washington University in St. Louis. I am also an

Editor of the scholarly publications Plant Cell Reports and Plant Cellular & Developmental Biology, Rapid Communications and am a co-editor for The Plant Cell and the author or co-author of more than 50 research publications in the field of genetics, plant molecular biology and crop transformation.

I have read and understood the invention described in the U.S. patent application of Robert T. Fraley and Stephen G. Rogers entitled "Chimeric Genes For Transforming Plant Cells Using Viral Promoter". The invention described therein involves in one aspect a method for transforming a plant cell to express a chimeric gene wherein the chimeric gene contains a viral promoter such as one selected from either the 35S or 19S promoter from Cauliflower Mosaic Virus (CaMV). The application also describes a chimeric gene, plant cell, plant transformation vectors and a differentiated dicotyledonous plant containing either the CaMV35S or CaMV19S promoter. The Cauliflower Mosaic Virus is known to only infect plants.

I am also familiar with U.S. Patent No. 4,536,475 which issued to Anderson on August 20, 1985. This patent purports to describe a chimeric gene capable of being expressed in plants using a Herpes Simplex virus thymidine kinase promoter to direct the expression of a gene that confers resistance to kanamycin (KAN).

During my 9 years involved in the study and research of transformation of plants, I have never encountered a reference that asserts that a promoter that is not normally expressed in plants can successfully promote expression of a gene linked thereto in plant cells, besides the Anderson patent. To my knowledge a non-plant promoter is not capable of performing at a functionable or measurable level in plant cells.

In order to test the assertions made in Anderson, plasmids were constructed for the purpose of testing the functionality of the herpes virus thymidine kinase (tk) promoter in plant cells and for the purpose of comparing the efficiency of the herpes virus thymidine kinase (tk) promoter to cause expression in plant cells with the efficiency of the cauliflower mosaic virus

(CaMV) 35S promoter in plant cells.

At my direction, Dr. Harry J. Klee, a molecular biologist at Monsanto, prepared the necessary plasmids. A chimeric gene containing the tk promoter, the neomycin phosphotransferase type II coding region and a 3' polyadenylation signal was purchased from Stratagene. This plasmid, pMC1NEO POLY A, was first described in Thomas and Capecchi (1987) and is marketed by Stratagene® as Cat. #213201 for the purpose of generating stably transformed mammalian tissues. This construction closely matches one described by Anderson in U.S. Patent No. 4,536,475. The plasmid used in Anderson is illustrated in Fig.4 of the '475 patent and described in Column 5, lines 9-12. It is stated in Anderson that the plasmid used, $pIPB_1$, could be obtained from Dr. B. Wold of the California Institute of Technology. Dr. Klee made repeated requests of Dr. Wold for the plasmid, but the plasmid was never provided. As described in Anderson, $pIPB_1$ contained the neomycin resistance gene from Tn5 cloned into the Herpes Simplex thymidine kinase gene adjacent the promoter. Plasmid pMClneo-PolyA also contains the Herpes Simplex thymidine kinase promoter adjacent the Tn5 neomycin phosphotransferase type II gene (hereinafter KAN) and additionally contains an enhancer sequence from polyoma virus Py F411 and a 3' poly (A)+ adenylation signal. The additional enhancer sequence and polyadenylation signal would only lead to enhance the performance of the tk promoter if it indeed worked in plant cells and would not be detrimental to the function of the tk promoter.

The KAN gene under the control of the tk promoter was inserted into an *Agrobacterium*-mediated plant transformation vector creating pMON16301. For comparison purposes, the KAN coding sequence was also placed behind the CaMV 35S promoter, creating pMON16300.

Plasmid pMON16300 contains the following DNA regions, in a clockwise direction, as indicated in Figure 1 which is attached hereto:

1. The 0.36 Kb PvuI to BclI fragment from the pTiT37 plasmid, which contains the nopaline-type T-DNA right border region (Fraley et al. 1985);
2. The chimeric kanamycin resistance gene engineered for plant expression to allow selection of the transformed tissue. The chimeric gene consists of the 0.35 Kb cauliflower mosaic virus 35S promoter (P-35S) (Odell et al. 1985), the 0.83 Kb neomycin phosphotransferase typeII gene (KAN), and the 0.26 Kb 3'-non-translated region of the nopaline synthase gene (NOS 3') (Fraley et al. 1983);
3. The 0.45 Kb ClaI to DraI fragment from the pTi15955 octopine Ti plasmid, which contains the T-DNA left border region (Barker et al. 1983);
4. The 0.75 Kb segment containing the origin of replication from the RK2 plasmid (ori-V) (Stalker et al. 1981);
5. The 3.0 Kb SalI to PstI segment of pBR322 which provides the origin of replication for maintenance in *E. coli* (ori-322), and the *bom* site for the conjugational transfer into the *Agrobacterium tumefaciens* cells; and
6. The 0.93 Kb fragment isolated from transposon Tn7 which encodes bacterial spectinomycin/streptomycin resistance (Spc/Str) (Fling et al. 1985), and is a determinant for selection in *E. coli* and *Agrobacterium tumefaciens*.

Plasmid pMON16301 contains the same DNA regions as pMON16300 except the chimeric kanamycin resistance gene, 35S/KAN/NOS 3', has been replaced with the 1.16 Kb XhoI to HindIII fragment from pMC1neo-PolyA which contains the chimeric gene with the Herpes Simplex thymidine kinase promoter and the enhancer sequence from polyoma virus Py F411 (P-TK), the Tn5 neomycin phosphotransferase type II gene (KAN), and the 3' poly(A)+ adenylation signal (poly A) as described above. Plasmid pMON16301 is illustrated in Figure 2 which is attached hereto.

The pMON16300 and pMON16301 vectors were then mobilized into the ABI *Agrobacterium* strain. The ABI strain is the A208 *Agrobacterium tumefaciens* carrying the disarmed pTiC58 plasmid pMP90RK (Koncz and Schell 1986). The Ti plasmid does not carry the T-DNA phytohormone genes, and the strain is therefore unable to cause the crown gall disease. Mating of vectors into ABI

strain is done by the triparental conjugation system using the helper plasmid pRK2013 (Ditta et al. 1980). When the plant tissue is incubated with the ABI conjugate, the T-DNA segment from the right border to the left border region of the vector is transferred to the plant cell by the vir functions encoded by the disarmed pTiC58 plasmid. The T-DNA is inserted into the plant chromosome, and the pTiC58 Ti plasmid remains in the *Agrobacterium*. For use as a negative control, a vector lacking the KAN gene, pMON813, was also mobilized into the A208 *Agrobacterium tumefaciens* strain carrying the disarmed pTiC58 plasmid pMP90RK. As a positive control, a vector containing the KAN gene, pMON977, was also mobilized into the A208 *Agrobacterium tumefaciens* strain. Plant tissue transformed with a vector containing pMON977 has previously been shown to be capable of growth in the presence of kanamycin in our laboratories.

The vectors 16300 and 16301 were then transformed into tobacco cells according to the following protocol. Leaves from 1 month old Samsun tobacco plants were surface sterilized in 10% chlorox with a few drops of detergent, rinsed 3 times with sterile distilled water, and then the outside margin, leaf tip, and mid-rib was removed and discarded. The internal leaf tissues were then cut into squares of 1/2 cm per side and precultured for 1 day. After the preculture, the explants were infected with the appropriate strain of *A. tumefaciens* carrying the vectors described, and cocultured on regeneration medium for 3 days. During this time, the bacteria bound to the plant cells around the wounded edge of the explant, and the gene transfer process occurred.

After the transformation step, the explants were transferred to regeneration/selection medium. The regeneration/selection was performed under two different conditions. The first set of conditions contained 500 μg/ml carbenicillin to kill the bacteria and 300 μg/ml kanamycin in the regeneration/selection media. This set of conditions is the standard selection protocol used routinely in our labs for production of large numbers of transgenic plants. The second set of conditions contained 500 μg/ml carbenicillin to kill the bacteria and 100 μg/ml kanamycin in the regeneration/selection media. This

second set of conditions provides a 3 times lower stringency selection media that would permit even poor KAN gene function to be detected. Other media and culture conditions were as previously described in Horsch et al., Science 227:1229-1231 (1985). This experiment is designed to reveal even marginal performance of a weak, but functional KAN gene construction. During the next 4 weeks, the transformed cells grew into callus or began to differentiate into shoots via organogenesis. Results were scored at 4 weeks post-inoculation.

The experiment was conducted twice, independently, with identical results obtained each time. Each treatment included 200 replicate leaf explants, the same number as a typical production run to produce 10 to 20 transgenic tobacco plants or to compare performance of other vectors.

The results of this experiment are best shown in Figure 3 and Figure 4 which are attached hereto. Figure 3 illustrates the results of selection under routine conditions; 300 μg/ml kanamycin. In the negative controls which contain no KAN gene, the kanamycin almost completely inhibits growth of callus and shoots of the transformed tobacco cells. The selection plates containing plant cells transformed with the tk/KAN vector also exhibit almost complete inhibition of growth of the transformed tobacco cells. In stark contrast, the selection plates containing plant cells transformed with the CaMV35S/KAN vector exhibit abundant callus and shoot growth on all leaf discs. Figure 4 illustrates the results of selection under less stringent selection conditions; 100 μg/ml kanamycin. Negative control leaf discs transformed with a vector lacking a KAN gene produce some limited growth of callus and shoots. Similarly, leaf discs transformed with the tk/KAN gene also produce some limited growth of callus and shoots at this level of kanamycin. The amount of growth of callus and shoots in the tk/KAN containing leaf discs is very similar to the amount of growth exhibited in the negative control leaf discs. The leaf discs transformed with the CaMV35S/KAN gene show substantially greater callus and shoot growth than either the negative control or the tk/KAN containing leaf discs.

The results obtained by using a tk/KAN gene to cause expression in plant

cells were virtually indistinguishable from the results exhibited by plant cells serving as a negative control. The conclusion to be drawn is that the tk promoter does not promote functional expression or measurable performance of a gene adjacent thereto beyond background levels obtained in control plant cells.

In the alterative, if one were to assume that a viral promoter, such as the tk promoter, is capable of causing expression in plant cells at some level, albeit a much lower one, the experiments described above illustrate the unexpected result that a promoter from Cauliflower mosaic virus is capable of substantially greater levels of expression in plants. This substantial and significant increase in the functional expression and measurable performance of the CaMV promoter is unexpected in view of the invention described in the Anderson patent.

The useful transformation of plant cells was dramatically improved by the invention as described in the Fraley and Rogers patent application.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statement may jeopardize the validity of the above-identified application or any patent issuing thereon.

7 Dec 90
Date

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